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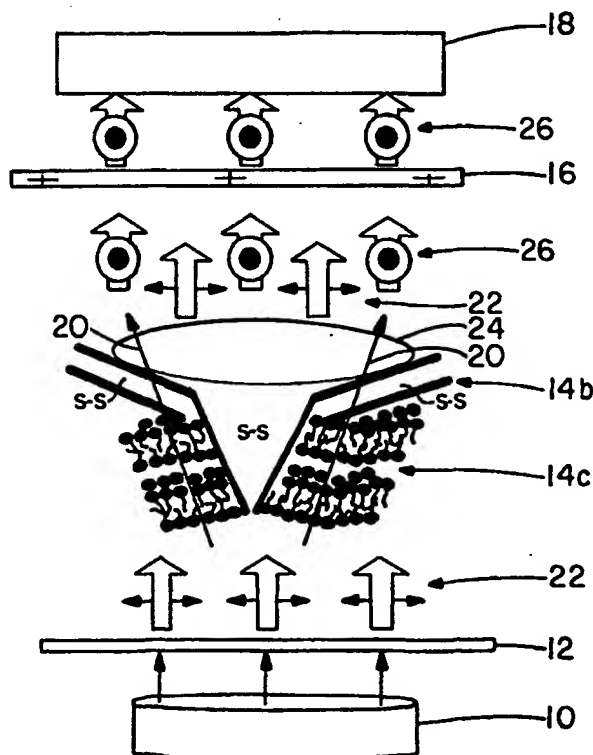
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(54) Title: DETECTION OF LIGANDS WITH SIGNAL AMPLIFICATION

(57) Abstract

A system for the detection of ligands comprising at least one receptor and an amplification mechanism coupled to the receptor wherein an amplified signal is produced as a result of receptor binding a ligand. Examples of suitable amplification mechanisms include antibody-embedded liquid crystalline materials; use of alpha-2-macroglobulin to encage an enzyme, whereby the enzyme is separated from its substrate by a receptor; and a receptor engineered to inhibit the active site of an enzyme only in the absence of a ligand. Also provided are methods for the automatic detection of ligands.



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DETECTION OF LIGANDS WITH SIGNAL AMPLIFICATION

TECHNICAL FIELD OF THE INVENTION

5 The present invention generally relates to the detection of a ligand by a receptor. More specifically, the present invention relates to highly specific receptors and the incorporation of these receptors into an amplification mechanism for the rapid and automatic detection of the ligand, particularly pathogens and/or their toxins.

10 BACKGROUND OF THE INVENTION

 The detection of a ligand by a receptor (for example, detection of a pathogenic agent such as a microbe or toxin by an antibody; or detection of an antibody in blood by another antibody; or binding of a chemical toxin, such as nerve gas, to its receptor) is
15 important in the diagnosis and treatment of individuals exposed to disease-causing agents. Early detection of pathogenic agents can be a great benefit in either disease prophylaxis or therapy before symptoms appear or worsen.

 Every species, strain or toxin of a microbe contains unique surface ligands.
20 Using molecular engineering and/or immunological techniques, receptor molecules, such as antibodies, can be isolated that will bind to these ligands with high specificity. Methods have also been developed where receptors, such as antibodies, are linked to a

signaling mechanism that is activated upon binding. Heretofore, however, no system has been developed that can quickly and automatically detect and amplify a receptor signal coming from the binding of a single or a low number of ligands. Such a system is imperative for rapid and accurate early detection of ligands.

5

Most available diagnostic tests are antibody based, and can be used to detect either a disease-causing agent or a biologic product produced by the patient in response to the agent. There are currently three prevailing methods of antibody production for recognition of ligands (antigens): polyclonal antibody production in whole animals with
10 recognition for multiple epitopes, monoclonal antibody production in transformed cell lines with recognition for a single epitope (after screening), and molecularly engineered phage displayed antibody production in bacteria with recognition of a single epitope (after screening). Each of these receptor systems is capable of binding and identifying a ligand, but the sensitivity of each is limited by the particular immunoassay detection
15 system to which it is interfaced.

Immunoassays, such as enzyme-linked immunosorbent assay (ELISA), enzyme immunoassay (EIA), and radioimmunoassay (RIA), are well known for the detection of antigens. The basic principle in many of these assays is that an enzyme-, chromogen-,
20 fluorogen-, or radionucleotide-conjugated antibody permits antigen detection upon antibody binding. In order for this interaction to be detected as a color, fluorescence or radioactivity change, significant numbers of antibodies must be bound to a correspondingly large number of antigen epitopes.

Thus, there is a need for a system that rapidly, reliably, and automatically detects ligands, especially when present in very small quantities and consequently provides a measurable signal.

5

SUMMARY OF THE INVENTION

It is therefore an object of the present invention to provide a system that will detect a ligand with high sensitivity and high specificity.

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It is another object of the present invention to provide a system that will amplify a signal produced by the binding of a ligand to a receptor.

It is another object of the present invention to provide a caged enzyme amplification mechanism.

15

It is yet a further object of the present invention to provide a system that will activate an enzyme inactivated by a bound receptor.

It is still a further object of the present invention to provide a system that will
20 distort a liquid crystal array upon the binding of a ligand to a receptor.

It is still yet a further object of the present invention to provide a detection device that will continuously monitor the environment or the body and signal its possessor when a ligand is present.

5 In general, the present invention provides a system for the detection and amplification of ligands, such as pathogenic agents, comprising at least one receptor and an amplification mechanism coupled to that receptor, wherein an amplified signal is produced as a result of the receptor binding the ligand.

10

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A is a schematic representation of the lamellar structure of a lyotropic liquid crystal formed by alternating layers of water and biphilic molecules.

15 FIG. 1B is a schematic representation of the amplification mechanism with a receptor inserted into the lyotropic liquid crystal.

FIG. 1C is a schematic representation of the amplification mechanism with the specific ligand bound to its receptor causing deformation of the liquid crystal and
20 alteration of the transmission of polarized light.

FIG. 2A is a schematic representation of a caged enzyme amplification mechanism in which one or more receptors blocks the channel leading to the enzyme in the absence of a ligand.

5 FIG. 2B is schematic representation of a caged enzyme amplification mechanism in which a ligand binds to the receptor causing distortion of the linker region and the unblocking of the channel leading to the enzyme.

FIG. 2C is a schematic representation of a caged enzyme antibody amplification
10 mechanism in which, once activated by ligand binding to receptor, the channel leading to the enzyme remains open and multiple substrates can interact with the enzyme.

FIG 3A is a schematic representation of an enzyme inactivated by a molecularly-engineered receptor.

15

FIG. 3B is a schematic representation of the binding of a ligand to the molecularly-engineered receptor causing dissociation of the receptor from the enzyme, exposing the active site of the enzyme to the substrate.

20 FIG. 3C is a schematic representation of substrate conversion by the enzyme, after picogram levels of ligand bind to the receptor.

FIG. 4A is a photograph showing the birefringence of polarized light through a liquid crystal-receptor array in response to binding of a specific ligand to the receptor.

FIG. 4B is a photograph showing the absence of birefringence of polarized light
5 through a liquid crystal-receptor array in a reaction mixture containing liquid crystal, receptor and PBS.

FIG. 4C is a photograph showing the absence of birefringence of polarized light
through a liquid crystal-receptor array in a reaction mixture containing liquid crystal,
10 receptor and an irrelevant ligand.

FIG. 5 is a graph showing the quantitative analysis of the birefringence of polarized light through an liquid crystal-receptor array of the ligand detection system for selective receptor-ligand binding, as compared to non-selective receptor-ligand binding
15 and background receptor-ligand binding.

FIG. 6A is a graph showing enzymatic activity of elution fractions containing an alpha-2-macroglobulin-trypsin complex.

20 FIG. 6B is a graph showing the protein concentration (ug/ml) of elution fractions containing an alpha-2-macroglobulin-trypsin complex.

FIG. 6C is a graph showing the effect of the concentration of caged trypsin on detectable enzymatic activity over time (min.).

FIG. 6D is a graph showing the effect of the concentration of caged thrombin on
5 detectable enzymatic activity over time (min.).

FIG. 6E is a graph showing the stability of caged trypsin over time (days).

FIG. 7A is a graph showing luciferase activity (Relative Light Units) over time
10 (sec.).

FIG. 7B is a graph showing luciferase activity (Relative Light Units) as a function of luciferase concentration (pg/ul).

15 DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

In the present invention, ligand-specific receptors are interfaced with an amplification mechanism such that a receptor-ligand interaction changes the conformation of the receptor and produces a signal. Amplification preferably occurs
20 through a colorimetric, fluorometric or birefringent shift that can be photometrically detected. The detected signal may then be electronically amplified to automate the system.

Ligand Detection Component

Any receptor, such as antibodies or biologic/biologically engineered receptors for ligands, can be incorporated into the device as long as binding of the ligand to the receptor causes a detectable distortion of the receptor. For example, any type of monospecific antibody (polyclonal, monoclonal, or phage displayed) can effectively function as a receptor, and thus each of those antibody types will be described in the following paragraphs. Although phage-displayed antibodies can be expeditiously modified for identification of new ligands and are used as receptor examples in this patent application, any physically-distortable receptor-ligand interaction is appropriate for the detection component.

Polyclonal antibodies: Antibody-based antigen detection has been exploited for several decades. Injection of a purified ligand (antigen) into a host animal stimulates the immune system to produce an array of antibodies against various reactive sites on the antigen. Since several lymphocytes are responding to different antigenic epitopes, a multi-specific antibody cocktail (polyclonal) is created and can be purified for antigen detection.

Monoclonal antibodies: Antibody-producing spleen cells (B lymphocytes) are fused with immortalized myeloma cells to create hybridomas which provide nearly infinite quantities of antibody with a single, defined specificity. Interstrain and even interspecies hybrids of these 'monoclonal' antibodies can be generated through genetic

engineering techniques. These highly specific antibodies have significant therapeutic potential, as evidenced by the U.S. Food and Drug Administration's approval of the use of mouse-human chimeric antibodies for treatment of selected diseases.

5 Phage-displayed mono-specific antibodies: Phage-displayed techniques will be used to isolate single chain chimeric antibodies to various pathogenic agents. The genomic DNA of the B lymphocyte contains the code to produce an antibody to virtually all possible ligands (antigens). In a phage displayed antibody system (PDA), DNA encoding a single chain chimera of the native antibody's hypervariable ligand-binding
10 region is synthesized by joining DNA encoding an antibody heavy chain and DNA encoding an antibody light chain and inserting therebetween DNA encoding a linker region. The desired amino acid sequence of the linker region depends on the characteristics required for any given amplification mechanism. The linker region may have to be able to interact and/or bond to a protein or other substance. Therefore, the
15 polypeptide sequence may have to have, for example, a particular conformation, specifically placed functional groups to induce ionic or hydrogen bonds, or a hydrophobicity that is compatible with the amplification mechanism. Regardless of the type of amplification mechanism, however, the linker region plays a critical role in interfacing the amplification mechanism to the receptor.

20

The DNA, preferably human or mouse, encoding the single chain chimeric antibody is cloned into a bacteriophage (phage) vector using well-known techniques (Marks et al., J. Mol. Bio. Vol. 222:581 (1991); Griffiths et al., EMBO J. 12:725 (1993);

and Winters et al., *Ann. Rev. Immunol.* 12:433 (1994)), incorporated herein by reference. The single chain chimeric antibodies then become displayed on the surface of a filamentous phage with the hypervariable antigen-binding site extended outward.

- 5 After the addition of ligands, phage that are reactive against non-targeted ligands are subtracted from the phage library using known techniques (Marks et al., *J. Mol. Bio.* Vol. 222:581 (1991); Griffiths et al., *EMBO J.* 12:725 (1993); and Winters et al., *Ann. Rev. Immunol.* 12:433 (1994)), incorporated herein by reference. The remaining phage are reacted with their specific ligand and phage reactive with that specific ligand eluted.
- 10 Each of these phage are then isolated and expressed in a bacterial host, such as *Escherichia coli* (*E. coli*) to produce a large quantity of phage containing the desired surface-displayed antibody. Each of the aforementioned methods relating to synthesizing and cloning DNA, subtracting phages, isolating and expressing phages and recovering viral DNA are well known and fully described by Marks et al., *J. Mol. Biol.*
- 15 (1991); Griffiths et al., *EMBO J.* 12:725 (1993); and Winters et al., *Ann. Rev. Immunol.* 12:433 (1994), all of which are incorporated herein by reference.

Amplification Component

- 20 Any mechanism that permits detection of ligand-receptor complex formation functions as an amplifier and can be incorporated into the device. Three amplification mechanisms are proposed. First, a liquid crystal will amplify the distortion caused when a ligand binds to a receptor. Second, an enzyme will be placed in a biologic cage and a

receptor will be attached to the biologic cage to prevent enzyme-substrate interaction. Ligand attachment to the receptor will open a substrate channel, resulting in enzyme-substrate interaction, and thus permitting detectable levels of reaction product. Third, the linker region of a receptor, such as a phage-displayed antibody, will be engineered to
5 bind and inhibit the active site of an enzyme. Dissociation of the receptor-enzyme complex occurs upon formation of an receptor-ligand complex, such as an antigen-antibody complex, and results in activation of the enzyme and generation of product.

Liquid Crystal: A liquid crystal is a state of matter in which molecules exhibit
10 some orientational order but little positional order. This intermediate ordering places liquid crystals between solids (which possess both positional and orientational order) and isotropic fluids (which exhibit no long-range order). Solid crystal or isotropic fluid can be caused to transition into a liquid crystal by changing temperature (creating a thermotropic liquid crystal) or by using an appropriate diluting solvent to change the
15 concentration of solid crystal (creating a lyotropic liquid crystal). Lyotropic liquid crystals will be used for our amplification system.

As seen in Fig. 1A, most lyotropic liquid crystals, designated generally by the numeral 1, are formed using water 2 as a solvent for biphilic molecules 3, for example,
20 molecules which possess polar (hydrophilic) aliphatic parts 4 and apolar (hydrophobic) aliphatic parts 5. When water 2 is added to a biphilic molecule 3, such as the cationic surfactant cetylpyridinium chloride [$C_{21}H_{33}ClN$], a bilayer 6 forms as the hydrophobic regions coalesce to minimize interaction with water 2 while enhancing the polar

component's interaction with water. The concentration and geometry of the specific molecule define the supramolecular crystalline order of the liquid crystal. The molecules can aggregate into lamellae as well as disk-like or rod-like micelles that form a nematic or cholesteric phase. The $C_{21}H_{38}CIN$ forms a lamella of alternating layers of water and biphilic molecules. An orientational order is created by the alternating layers of water and biphilic molecules and thus the liquid crystal is opaque (exhibits optical anisotropy) to polarized light 7 provided by a light source located perpendicular to the plane of the liquid crystal.

10 Most biologic receptors possess both hydrophilic and hydrophobic regions and thus readily incorporate into biphilic lyotropic liquid crystals. Additionally, the inactivated receptors do not destroy the optical anisotropy of the liquid crystal and therefore, the receptor-enriched liquid crystal remains opaque to polarized light (Fig. 1B). However, optical anisotropy is disrupted when receptor conformation shifts as 15 during the formation of the receptor-ligand complex (Fig. 1C). The elasticity of the liquid crystal enhances the local distortions in the vicinity of the receptor-ligand complex, and expands it to an optically detectable, supramicron scale. Biologic materials can be detected on the surface of thermotropic liquid crystals (V.K. Gupta et al., Science 279:277-2080, 1998). However, lyotropic liquid crystals readily incorporate 20 ligand-specific receptors, and are thus clearly superior for detection of biologic molecules.

Configurations of Ligand Detection System

By way of example, one envisioned application of the present invention is in a
5 multiwell system. Each well of the system would contain PDAs to a specific ligand,
such as a pathogenic microbe, interfaced with an amplification mechanism of the present
invention. When the microbial agent interacts with the antibody, the resulting antibody
distortion triggers the amplification mechanism. Preferably, the amplified signal is then
transduced into a perceptible signal. Accordingly, it is envisioned that such a system
10 could be placed in a physician's office, and be used in routine diagnostic procedures.
Alternatively, such a system could be placed on or near soldiers in battle, and the
invention used to alert the soldiers to the presence of a toxic agent. It is further
envisioned that a multiwell system, although it can be used with other embodiments,
such as the luciferase or the caged enzyme described hereinbelow, is preferably used in
15 conjunction with the liquid crystal embodiment described herein.

Thus, in one embodiment of the present invention, shown schematically in Figs.
1B and 1C, a lyotropic liquid crystalline material is used as an amplification mechanism.
As shown in Fig. 1B, the device consists of a light source 10, an initial polarizer 12, with
20 the direction of polarization in the plane of the figure, a pathogen detection system 14a,
comprising nonspecific antibodies 14b embedded in biphilic, lyotropic liquid crystalline
material 14c, a secondary polarizer 16, with the direction of polarization perpendicular
to the plane of the figure, and a photodetector 18.

In operation, the initial polarizer 12 organizes a light stream 22 that is linearly polarized in the plane of the figure. The optical axis 20 of the inactivated device is perpendicular to the pathogen detection system 14a, and thus no birefringence of the transilluminating linearly polarized light stream 22 occurs. Since the polarization
5 direction of the secondary polarizer 16 is perpendicular to the transilluminating linearly polarized light 22, the secondary polarizer prevents light from reaching the photodetector 18.

Binding of a ligand 24, such as a microbe, to the receptor 14b, such as an
10 antibody, distorts the liquid crystal 14c, induces birefringence and thus causes the generation of detectable light. This activation process is illustrated in Fig. 1C. The receptor (antibody) 14b is embedded in the biphilic, lyotropic liquid crystal 14c. The spacial distortion caused by the formation of the antigen-antibody complex is transmitted to the contiguous liquid crystal 14c. The elastic characteristics of the liquid
15 crystal permit the distortion to be transmitted over a region much larger than the size of the receptor-ligand complex. This allows the use of the standard optical phenomenon of birefringence to detect distortions caused by the receptor-ligand complex. The altered liquid crystalline order tilts the optical axis 20 and induces birefringence. In other words, the incident polarized light 22 gives rise to two refracted light waves: the
20 ordinary wave and the extraordinary wave with the mutually orthogonal polarizations (see, Max Born and E. Wolf, Principals of Optics, Sixth edition, Pergaman Press, Oxford, 1980), incorporated herein by reference. Thus, there is a portion of light 26 in which the optic vibrates in the direction of the secondary polarizer 16. The secondary

polarizer (analyzer) 16 allows this portion of the light to pass to the photodetector 18. The detected change or amplification in light intensity can be transduced electronically into a perceptible signal.

5 In another embodiment of the present invention, shown schematically in Figs 2A-2C, enzyme-substrate interactions are exploited for amplification. With reference to Fig. 2A, an enzyme 30 is entrapped within a cage 32 to prevent premature interaction of enzyme 30 and a substrate 34. Substrate 34 can potentially gain access to enzyme 30 through one or more channels 36 transecting cage 32. Channel 36, however, is blocked
10 as indicated by arrow 37, by one or more receptors having dual binding capacities (diabodies) 38 in the absence of a pathogenic agent.

To produce a diabody, two antibodies are coupled, preferably according to the methods of Holliger et al. (Proc. Natl. Acad. Sci USA 90:6444, 1993), incorporated
15 herein by reference, to form diabody 38 which has two distinct antigen-binding regions 40 and 41, directed toward channel 36 and a ligand 48, respectively. Ligand-binding region 40 consists of a heavy chain immunoglobulin 42a and a light chain immunoglobulin 43a. Ligand-binding region 41 consists of a heavy chain immunoglobulin 43b and a light chain immunoglobulin 42b. Without intending to be
20 bound by any particular theory, it is believed that the binding of binding region 40 to one or more diabodies 38 to an epitope on cage 32 blocks channel 36 and prevents substrate-enzyme interactions. Thus substrate 34 is not processed under non-stimulated conditions.

With reference to Fig. 2B, when binding region 41 attaches to ligand 48, the linker regions 44 and 46 of diabody 38 are distorted so that channel 36 becomes unblocked and substrate 34 can pass through channel 36, indicted by arrow 49, and
5 interact with enzyme 30. Once activated by ligand 48, channel 36 remains open and multiple substrates 34 can enter and be acted on to produce products 34a and 34b which may be colored products that are chromophoric or flourescent. Either one or both products 34a and 34b are then detected or amplified, and may be transduced into a perceptible signal.

10

Although one skilled in the art would realize that various globular structures, preferably proteins, could be used as cage 32, alpha-2-macroglobulin is particularly well suited for acting as an enzyme cage because several enzymes, including trypsin and thrombin, will partially degrade alpha-2-macroglobulin, enter the protein, and become
15 entrapped merely by mixing the alpha-2-macroglobulin and enzyme.

Enzyme 30 can be any that can be entrapped in a molecular cage and that produces a detectable change of its substrate. One of ordinary skill in the art would realize that a myriad of enzyme-substrate pairs are detectable and therefore suitable.
20 Thrombin and trypsin, for example, are two preferred enzymes. A preferred substrate has a recognition site and a chromogen such that detectable colorimetric change occurs upon enzyme-substrate binding.

Linker regions 44 and 46 are engineered to allow substrate 34 to enter channel 36 upon binding of ligand 48 to antigen binding region 41. Linker regions 44 and 46 are preferably of moderate length. The skilled artisan would appreciate that if the length of linker regions 44 and 46 are too short or too long, then these regions may not distort
5 adequately to unblock channel 36. The preferred range of linker region length depends primarily on the nature of cage 32 and channel 36.

Linker regions 44 and 46 are also preferably bonded together by ionic, hydrogen, or other bonds. Otherwise, linker regions 44 and 46 would be susceptible to separation
10 and consequently inadequate distortion upon binding of a pathogenic agent.

An another embodiment of the present invention, represented schematically in Figs 3A-3C, also uses an enzyme-substrate amplification mechanism. As shown in Fig. 3A, a single chain chimeric antibody 50 is engineered such that linker region 52
15 specifically binds an enzyme 54 to inhibit the binding of a substrate 56 to enzyme 54, as indicated by arrow 58. In this embodiment, linker region 52 has a portion of its polypeptide sequence that is complementary to the active site of enzyme 54. Techniques for the determination of suitable complementary polypeptides are well known.

As shown in Fig. 3B, when a binding region 60 of antibody 50 binds a ligand 62,
20 a conformational change in linker region 52 results in the dissociation of antibody 50 from enzyme 54. Liberated enzyme 54 is now able to act on substrate 56. Each liberated enzyme can react with multiple substrates to provide an amplified signal. By

way of example only, a particularly preferred embodiment is depicted in Fig. 3C showing how an enzyme, such as luciferase, can be used to alter its substrate, in this example, the oxidation and dissociation of luciferin. This reaction produces a fluorescent species 57a that is detectable below picogram levels of reaction product, and
5 a non-fluorescent species 57b. Fluorescent species 57a is then detected or amplified and may be transduced into a perceptible signal. Enzyme 54 can be any enzyme that produces a detectable conversion of its substrate.

EXPERIMENTAL

10

Liquid Crystal Amplification Mechanism

A ligand detection system using a liquid crystal amplification mechanism was developed. A murine antibody to *E. coli* lipopolysaccharide (LPS), a surface antigen
15 found on Gram-negative bacteria, was obtained from a commercial source (Biodesign International, cat. # C61212M, clone #26-5, lot #5D1197) and diluted (1:10) with sterile phosphate-buffered saline (PBS) pH 7.2 to yield a 100 ng / μ l sample.

A late log phase culture of *E. coli* was grown in Brain Heart Infusion (BHI)
20 broth and washed free of growth medium with 0.9% sterile saline. Bacterial numbers (determined by optical density at 600 nm and colony-forming units (CFUs)) were

quantitated by extrapolation from growth curve data. Bacteria were then adjusted to 4.6×10^1 CFU per 5 μ l by dilution with sterile saline.

The lyotropic liquid crystalline solution was prepared in a lamellar phase, using
5 hexanol as a co-surfactant to help control the phase state of the mixture. Cetylpyridiniumchloride (CpCl) was combined with hexanol in the proportion hexanol/CpCl = 0.651 (w/w). The mixture was then diluted with a saline solution (1% NaCl in water) until 85% of the weight was saline. The resulting liquid crystal was lamellar but close to the micellar phase (Nastishin, Langmuir. Vol. 12, pp. 5011 - 5015,
10 1996), incorporated herein by reference. In this phase, the lyotropic liquid crystal is biphilic, and thus is capable of interacting with several different types of receptors.

The tested detection system was created by inserting a receptor (antibody) into the hexanol/CpCl/saline lyotropic liquid crystal. Thus, for each assay, 5 μ l of the
15 antibody (500 ng) solution, specifically reactive against *E. coli* (LPS), was added to 5 μ l of the lyotropic liquid crystalline solution and mixed. An experimental sample (5 μ l) was then added to an aliquot (10 μ l) of the liquid crystal-antibody mixture and mixed. The experimental samples were added to the antibody-liquid crystal mixture as follows: Sample A-5 μ l of *E. coli* (4.6×10^1 CFU), a specific bacteria that the system is designed
20 to detect; Sample B- 5 μ l of PBS, or Sample C-5 μ l (2×10^6 CFU) *S aureus*, an irrelevant bacteria that the system should not detect. The samples were centrifuged (3,500 x g; 5 sec.) to eliminate bubbles, and 10 μ l of the reaction mixture was placed on an ethanol-cleaned microscope slide, covered with an ethanol-cleaned glass cover slip, and the mixture was evaluated for birefringence using polarized light. The experimental
25 conditions and results are summarized in Table 1. Birefringence occurred only when the

antibody was bound to its specific antigen, with visually discernable changes in birefringence detected for bacteria concentrations as low as 46-460 CFU per 5 μ l.

TABLE 1

<u>Sample</u>	<u>Antibody (LPS)</u>	<u>E. coli</u>	<u>S. aureus</u>	<u>PBS</u>	<u>Liquid Crystal</u>	<u>Birefringence</u>
A	500 ng	4.6×10^1	---	---	5 μ l	YES
B	500 ng	---	---	5 μ l	5 μ l	NO
C	500 ng	---	2×10^6	---	5 μ l	NO

5 In other experiments, LPS antibody, *E. Coli* (2.7×10^7 CFU) and liquid crystalline material were reacted in a manner similar to the previous experiment, and representative photomicrographs (110X magnification; Figs. 4A, 4B and 4C) were evaluated with a Bio-Quant Image Analysis System. The image analysis was performed to quantitatively compare propagating light transmission when *E. coli*, PBS or *S. aureus*
 10 was evaluated by the receptor-ligand binding system. The photographic images were digitized and integrated optical density (IOD) automatically calculated according to the following formula:

$$\text{IOD} = \frac{(\Sigma - \log_{10})(\text{foreground})}{(\text{background})}$$

15

The resulting data, presented in Fig. 5, show that a profound increase in transmission of propagating light occurs when liquid crystals amplify the binding of antibody to *E. coli* (LPS).

20

Caged Enzyme Amplification Mechanism

The sensitivity of an alpha-2-macroglobulin amplification mechanism was demonstrated. Standard 2mg/100 μ l solution of human alpha-2-macroglobulin (α_2 M,
 25 Calbiochem Co., product number 441251), trypsin (Sigma Chemical Co., T-8003) and thrombin (Sigma Chemical Co., T-4648) were made by dissolving the protease or

antiprotease in 0.1 M HEPES buffer (pH 7.6). Equal volumes (100 μ l) of α_2 M and one of the enzymes were mixed, permitted to interact for ten minutes at room temperature and then cooled to 4°C. The 200 μ l sample was added to a gel filtration column (1 cm x 24 cm; 18.8 ml bed volume; 0.44ml/min flow rate; located in a 4°C cold room) packed with Sephadex G-100 to separate the caged enzyme from uncomplexed enzymes. Column eluent was collected in 1.0 ml fractions. Changes in light absorbance at 280 nm was measured to determine the protein concentration in each fraction and enzymatic activity was determined to identify those fractions containing the caged enzymes. The results of those measurements, shown in Figs. 6A and 6B, demonstrate that fractions 15-17 contained relatively pure samples of caged enzyme. Those fractions were used for the subsequent evaluations.

Small synthetic substrates, N-benzoyl-L-arg-p-nitroanalide and N-p-tosyl-gly-pro-arg-p-nitroanalide, were used to define the enzymatic activities of caged trypsin (Fig. 6C) and caged thrombin (Fig. 6D), respectively. While both systems exhibited dose-response characteristics, the caged thrombin exhibited greater sensitivity. The enzymatic activity did not degrade with time. The enzymatic activity of the caged trypsin was unchanged following six days of storage at 4°C (Fig. 6E). Similarly, caged thrombin activity was also stable when measured 24 hours following preparation.

Luciferase Amplification Mechanism

The exceptional sensitivity of a luciferase-based amplification mechanism was demonstrated using a Berthold Lumat Luminometer. Varying amounts of luciferase (4 mg/ml of 0.15% NaCl, 10mM HEPES, 1mM EDTA, 2 mM MgCl₂, 2 mM dithiothreitol; Sigma Chemical Co.) were added to the luminometer reaction chamber. The enzymatic reaction was initiated by rapid injection of 0.5 mM luciferin (Promega, E1483), 0.5 mM adenosine triphosphate (Sigma Chemical Co., A-7699), 5 mM MgSO₄, 1.0 mM dithiothreitol (Sigma Chemical Co.) in 50 mM HEPES buffer (pH 7.8) into the reaction chamber. Fig. 7B shows that detectable luciferase activity can be measured with an

enzyme concentration of only 0.0017 pg/ μ l, and linear increases in activity are observed with progressive elevations in enzyme concentration.

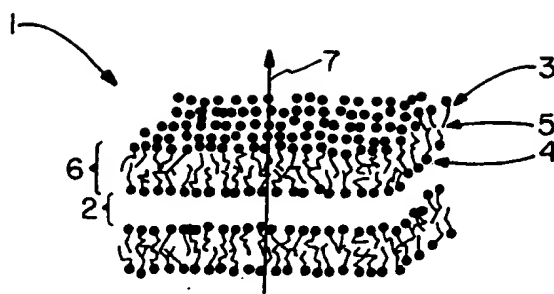
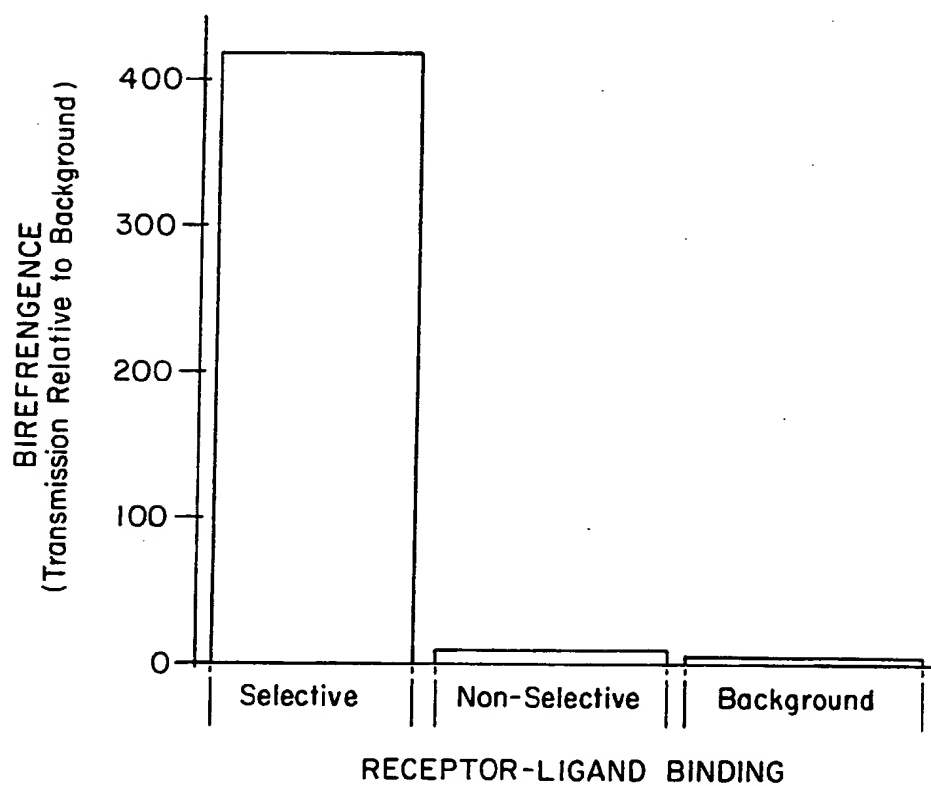
It is to be understood that any variations evident fall within the scope of the
5 claimed invention, and thus the selection of specific antibodies, caged enzymes,
receptor-inactivated enzymes or liquid crystals can be determined without departing
from the spirit of the invention herein disclosed and described. It should also be
understood that the present invention, while particularly suited for pathogen detection, is
intended to include the detection of any ligand. Moreover, the scope of the invention
10 shall include all modifications and variations that may fall within the scope of the
attached claims.

We Claim:

- 1 1. A system for the detection of ligands comprising:
2 at least one receptor; and
3 an amplification mechanism coupled to the receptor, said amplification
4 mechanism generating an amplified signal when the receptor binds to the
5 ligand.
- 1 2. The system of claim 1, wherein the receptor is an antibody selected from the
2 group consisting of monoclonal, polyclonal and molecularly engineered
3 antibodies.
- 1 3. The system of claim 1, wherein the ligand is a pathogenic agent.
- 1 4. The system of claim 1, wherein the amplification mechanism includes a liquid
2 crystalline material having the receptor incorporated therein.
- 1 5. The system of claim 4, wherein the amplified signal is generated by a change in
2 optical characteristics of the liquid crystalline material.
- 1 6. The system of claim 1, wherein the amplification mechanism comprises:
2 an enzyme 30 within a biologic/molecular cage 32; and
3 wherein the receptor 38 is bound to the biologic/molecular cage 32 and
4 wherein the amplified signal results from the enzyme acting upon a plurality of
5 substrate molecules once the receptor binds to the ligand.
- 1 7. The system of claim 6, wherein the biologic/molecular cage is a globular protein.
- 1 8. The system of claim 6, wherein the biologic/molecular cage is alpha-2-
2 macroglobulin.

- 1 9. The system of claim 6, wherein the receptor is an antibody selected from the
2 group consisting of monoclonal, polyclonal and molecularly engineered
3 antibodies.
- 1 10. The system of claim 6, wherein receptor binds to a specific ligand.
- 1 11. The system of claim 6, wherein the receptor is a diabody having a ligand-binding
2 region and a cage-binding region connected by a linker region.
- 1 12. The system of claim 6, wherein the receptor blocks the channel before ligand
2 binding, and wherein the cage has a channel through which the substrate
3 molecules pass after ligand binding.
- 1 13. The system of claim 6, wherein the receptor is a diabody, and wherein the ligand
2 is a pathogenic agent.
- 1 14. The system of claim 1, wherein the amplification mechanism includes an enzyme
2 having an active site for a substrate, and wherein the receptor inhibits substrate-
3 enzyme interaction prior to ligand binding, and wherein the enzyme is liberated
4 upon ligand binding, and wherein the amplified signal results from the substrate-
5 enzyme interaction.
- 1 15. The system of claim 14, wherein the receptor is an antibody and the ligand is
2 pathogenic agent.
- 1 16. The system of claim 14, wherein the amplified signal is fluorometric change.
- 1 17. The system of claim 14, wherein the enzyme is luciferase.

- 1 18. The system of claim 14, wherein the antibody is selected from the group
2 consisting of monoclonal, polyclonal and molecularly engineered antibodies.
- 1 19. The system of claim 14, wherein the receptor has a linker region that binds to the
2 active site of the enzyme prior to ligand binding.
- 1 20. A device for the detection and monitoring of the presence of ligands comprising:
2 multiple wells, each well having a predetermined receptor thereon; and
3 an amplification mechanism coupled to the predetermined receptor that is
4 activated upon binding of a specific ligand to its predetermined receptor.
- 1 21. The device of claim 20, wherein the receptor is an antibody.
- 1 22. The device of claim 21, wherein the antibody is selected from the group
2 consisting of monoclonal, polyclonal and molecularly engineered antibodies.
- 1 23. The device of claim 22, wherein the amplification mechanism generates an
2 amplified signal upon binding.
- 1 24. The device of claim 23, wherein said amplified signal is translated into a
2 perceptible signal.

FIG.-1AFIG.-5

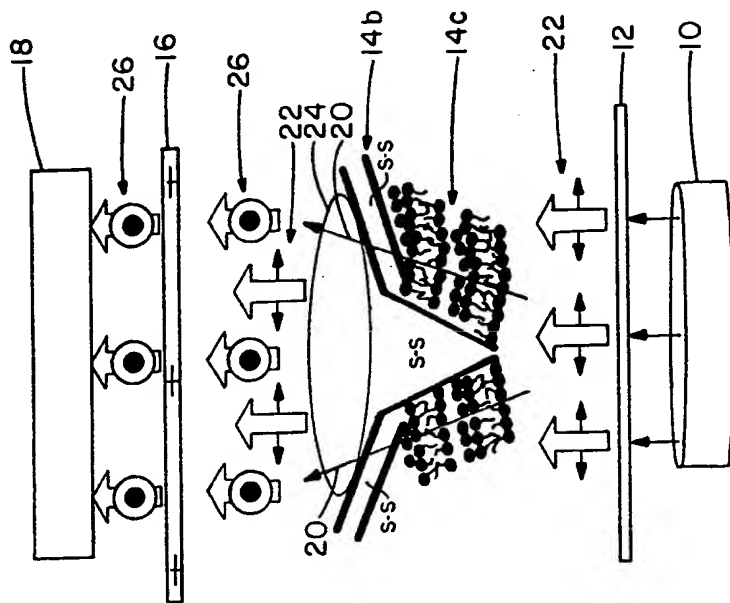


FIG. - 1C

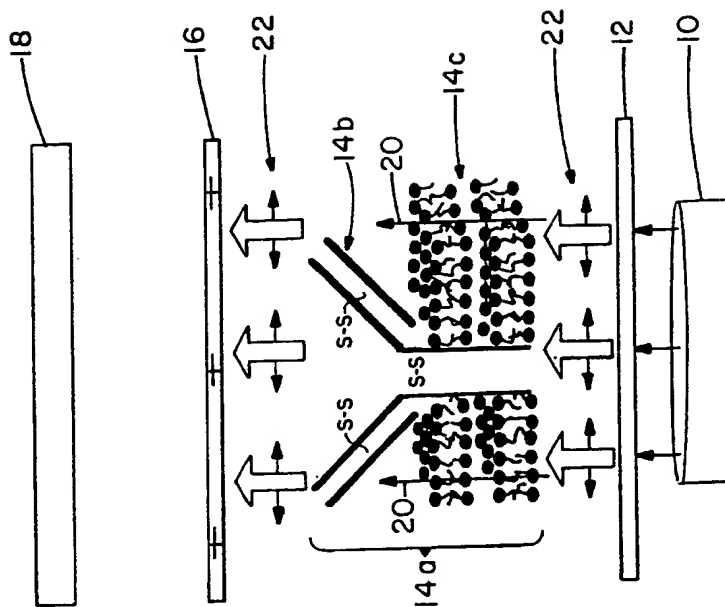
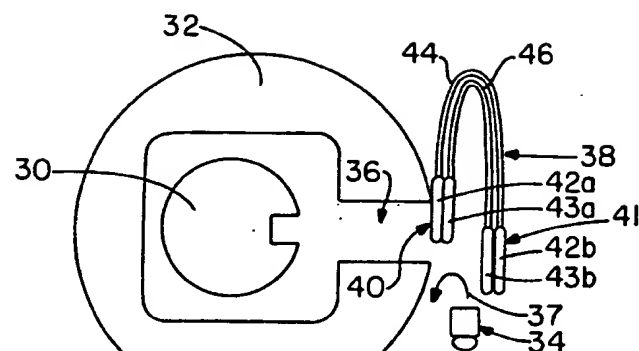
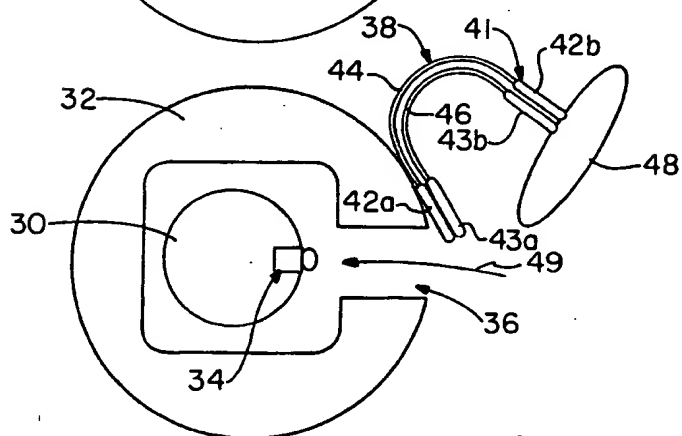
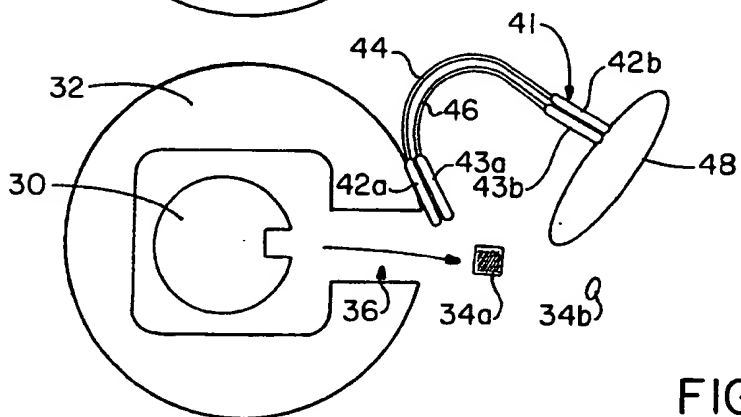
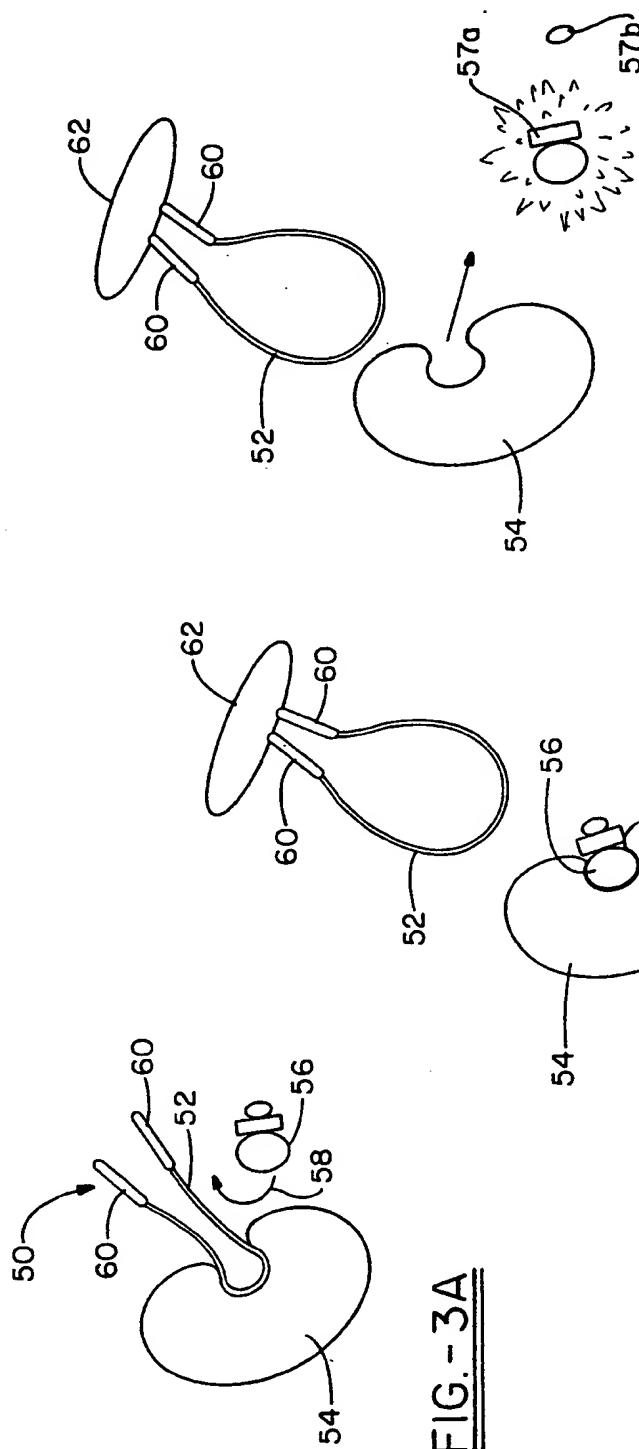


FIG. - 1B

FIG. - 2AFIG. - 2BFIG. - 2C



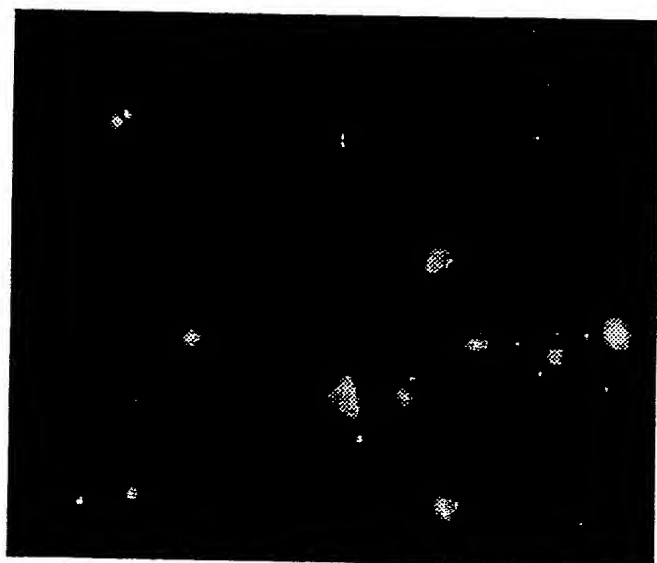


Fig. 4A

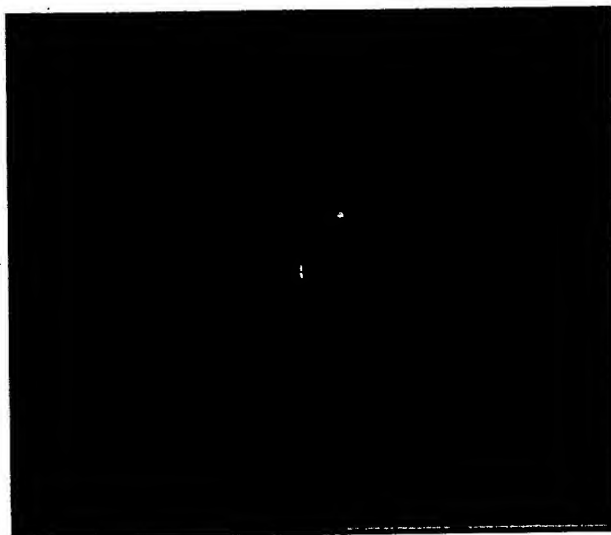


Fig. 4B

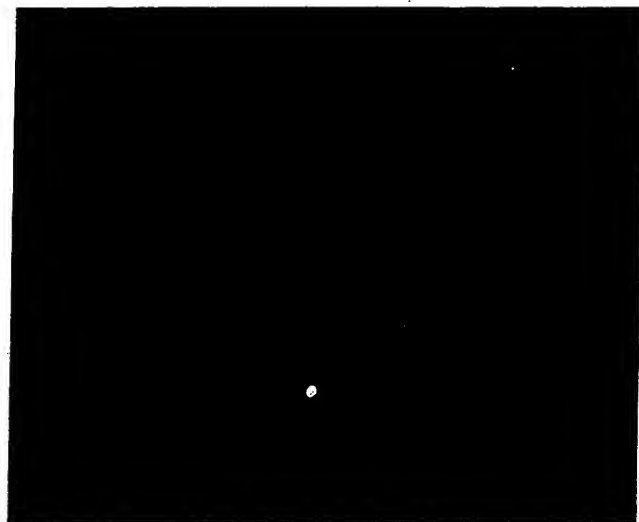
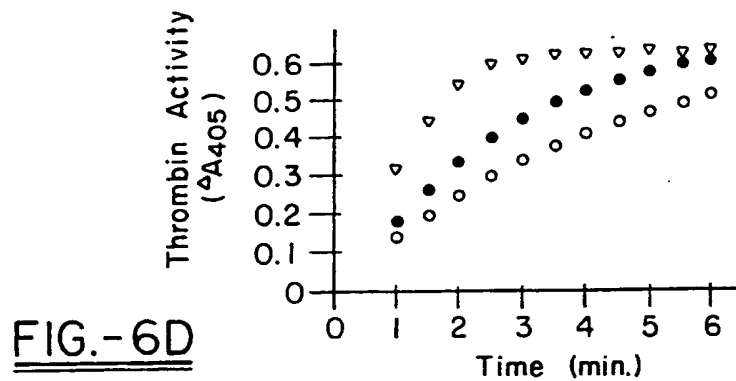
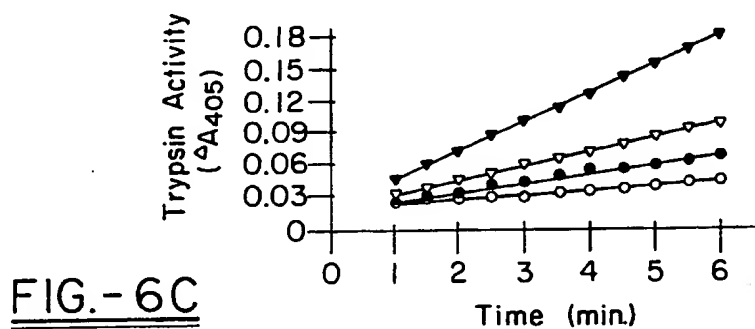
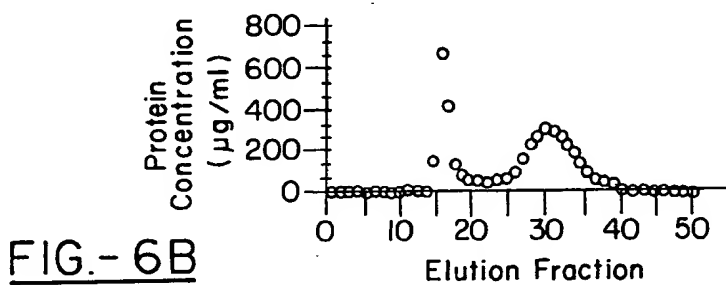
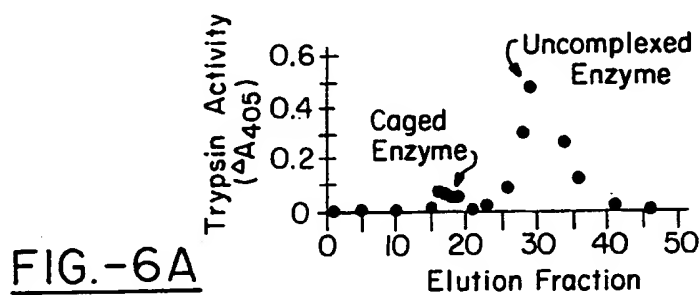
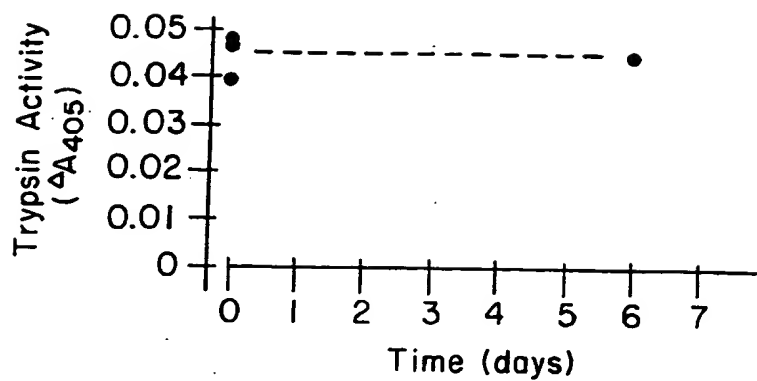
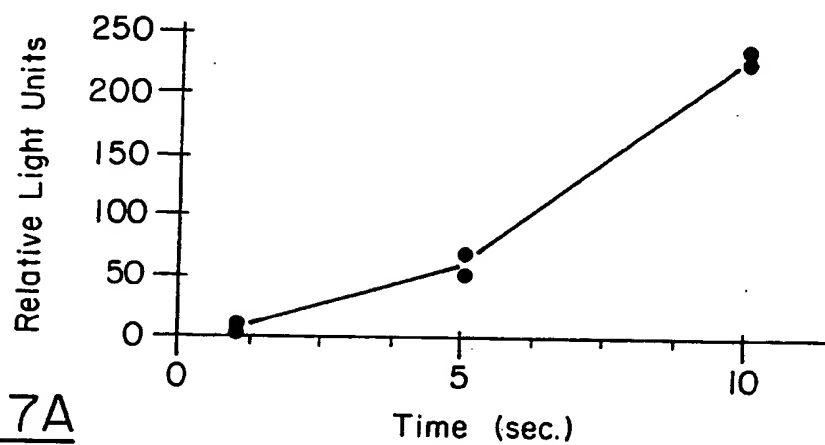
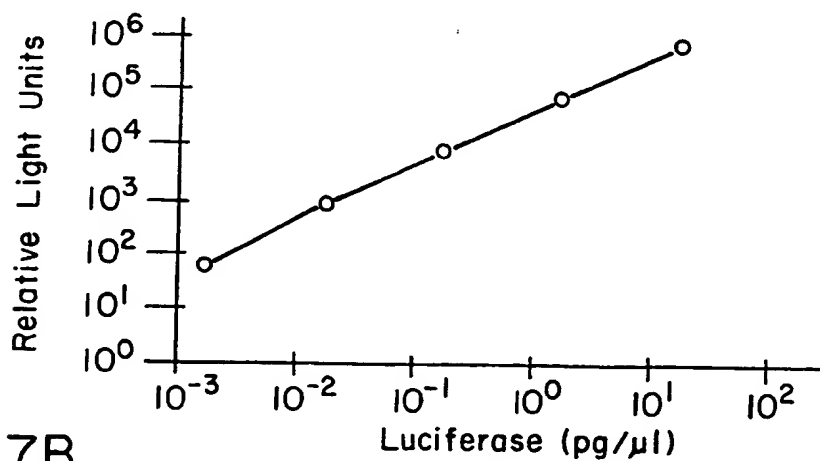


Fig. 4C



FIG.-6EFIG.-7AFIG.-7B

INTERNATIONAL SEARCH REPORT

national Application No
PCT/US 99/10413

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/543

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GUPTA, V.K. ET AL.: "Optical Amplification of Ligand-Receptor Binding Using Liquid Crystals" SCIENCE, vol. 279, 27 March 1998 (1998-03-27), pages 2077-2080, XP002115846 cited in the application the whole document	1-5, 20-24
A	R. SUTHERLAND ET AL.: "Surface effect immunoassay", STOCKTON PRESS, 1991; ISBN: 1-56159-020-7, NEW YORK, USA; PAGES 515-542 XP002115848 page 526, line 17 -page 529, line 10; table 18.2	1-5, 20-24
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "Z" document member of the same patent family

Date of the actual completion of the international search

20 September 1999

Date of mailing of the international search report

23. 11. 1999

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Authorized officer

Gundlach, B

INTERNATIONAL SEARCH REPORT

b International Application No
PCT/US 99/10413

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	R. SEABROOK AND T. ATKINSON: "Modification of Antibodies", STOCKTON PRESS, 1991; ISBN: 1-56159-020-7, NEW YORK, USA; PAGES 53-77 XP002115849 page 68, line 19 -page 73, line 10 ---	1,2
X	DE 36 17 710 A (OLYMPUS OPTICAL CO) 4 December 1986 (1986-12-04) the whole document ---	1-5
A	GUPTA, V.K. ET AL.: "Design of Surfaces for Patterned Alignment of Liquid Crystals on Planar and Curved Substrates" SCIENCE, vol. 276, 6 June 1997 (1997-06-06), pages 1533-1536, XP002115847 the whole document -----	1-5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/10413

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1, 20-24 (partially); 2-5 completely)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 99/10413

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1, 20-24 (in part) 2-5 (in full)

Detection of ligands for receptors by a change of optical characteristics of a liquid crystalline material.

2. Claims: 1, 20-24 (in part) 6-13 (in full)

Detection of ligands for receptors with the help of a caged enzyme.

3. Claims: 1, 20-24 (in part) 14-19 (in full)

Detection of ligands for receptors by activating an enzyme inactivated by a bound receptor.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/10413

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE 3617710 A	04-12-1986	JP 61271460 A	01-12-1986

